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Effect of Host Species on Hatchability of *Fasciola hepatica* and *Fasciola gigantica* Eggs from Sheep and Cattle

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ABSTRACT

Introduction: Successful development of free-living stages of parasitic helminths depends on larva ability to survive, develop, and hatch. In this study, we aimed to study the host role in the hatching process of *Fasciola* species. **Methods:** *Fasciola hepatica* and *Fasciola gigantica* eggs were collected from adult worms that originated from naturally infected sheep and cattle livers and were incubated at 26±1°C for 15 days. The percentage of hatched and developed eggs were obtained for each isolate under a light microscope. A polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) was applied to identify the *F. hepatica* and *F. gigantica* species. **Results:** Our findings showed no significant differences in the development rates of *F. gigantica* and *F. hepatica* eggs in sheep (69.32% and 72.71%) and cattle (73.56% and74.69%). However, the rates of hatched eggs of *F. gigantica* and *F. hepatica* originated from cattle (69.19% and 62.36%) were almost twice the rates in sheep (31.69% and 32.59%), indicating a significant difference. **Conclusion:** This study demonstrated that host species significantly affect the hatching of *Fasciola* eggs as the hatching rates of *F. gigantica* and *F. hepatica* originated from cattle were higher than those taken from sheep did not affect their larval development. Thus, in addition to environmental factors, the hatching phenomenon is influenced by host species.

INTRODUCTION

Parasitic helminths use different strategies to optimize transmission. The egg or larval stages of parasites deploy some strategies, i.e., by suppressing the host immune system to enhance their survival in the host, including protecting the parasite infective stages and inhibiting the aggressive immune effects in repelling the parasite eggs [1]. The primary factors that affect the hatching of free-living helminth eggs are temperature and humidity [2], though these conditions vary among different parasite species. Other important environmental factors contributing to the hatching and survival of helminths include pH, light, and nutrients [3]. In addition to the effect of environmental and parasitic factors in the hatching process, the host interactions with the parasite or immunity can also impose severe restrictions on this process [4-6]. Strong host responses to the trematodes egg by targeting the surface antigenic molecules or excretory/secretory compounds of juvenile or adult oncosphere could directly or interact with female worms and affect egg hatchability rate and volume [1, 7]. *F. hepatica* and *F. gigantica* are common parasites of ruminants, causing significant economic losses in the livestock industry. The geographic distribution of both species overlaps in many regions of Asia, such as Iran and Africa [8]. Human health problems caused by *Fasciola* species have changed the epidemiological features of the disease from a secondary zoonotic disease to a significant human parasitic disease [9, 10]. Both *Fasciola* species develop in a similar life cycle pattern and produce eggs in mammals' bile ducts. In suitable environmental conditions, once shed in the host feces, the embryonic cells in eggs grow and develop into miracidia and hatch. Miracidia penetrate actively into the suitable mollusk and evolve to the sporocysts, redia, and cercaria stages. Finally, the free motile cercariae emerge from snails and encyst on the vegetation into metacercariae. Environmental factors such as light, temperature, pH, sodium chloride concentration, and the incubation period can affect the development and hatching of *Fasciola* eggs [11-14].

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Studying various parasite life cycle stages can open new ground for control and prevention programs to reduce the final host's infection risk [15]. Some reports have indicated the impact of environmental factors on the hatching *Fasciola* spp eggs [12, 16]; however, the data on the host role in larva development and egg hatching is limited. Ashrafi et al. (2006) showed that the age of the host, helminth, and method of preparing the specimens affect the adult *Fasciola* eggs morphological characteristics [17]. Also, Lambert et al. (2015) demonstrated the host role in egg development and hatchability by detecting the egg-specific antibody responses [1]. They demonstrated the female worm-host interactions could potentially affect the quality and hatching rate of shedding eggs.

This study aimed to compare the hatchability of fertile intrauterine eggs of *F. hepatica,* and *F. gigantica* originated from bovine and ovine hosts.

MATERIALS AND METHODS

Egg hatch assay. Naturally-infected ovine and bovine livers of *F. hepatica* and *F. gigantica* with light primary infections were obtained from municipal abattoirs of Ahvaz, southwest of Iran. The parasites were removed only from livers with low-intensity infection (less than 30 adults per host); in cattle with no calcifications or stones in their bile ducts. The mature liver flukes (five samples of each host species) were identified based on morphological and molecular criteria. In this way, after the morphological diagnosis of the adult worms, the anterior third part of the trematode body, including the egg-filled uterus down to about 1 mm post ventral sucker containing the high rates of gravid eggs, was carefully dissected. The remainder of worms were stored at 70% alcohol in -20ºC. Tissues associated with the anterior third part of the body were crushed, and the eggs were harvested by passing through a 100-mesh sieve. The collected eggs were washed several times in 0.9% normal saline followed by centrifugation and were individually transferred to the separate dark containers with holes on the lead and labeled. All the containers were kept in the incubator under a suitable temperature (26±1ºC) and moisture for 15 days. After the incubation period, the eggs were exposed to light for one hour to stimulate the hatching. Finally, the number of hatched, embryonated, and unembryonated eggs were counted under a light microscope. The hatching assays were repeated three times for each sample.

Data and statistical analysis. The percentage of developed and hatched eggs of *F. hepatica* and *F. gigantica* in the sheep and cattle were calculated using the following formulas: Percentage of development = (number of developed eggs/total number of eggs) x 100 and Percentage of hatching = (number of hatched eggs/total number of eggs) x 100.

The number of developed eggs is the sum of hatched (operculum opened without miracidium) and embryonated eggs (miracidium inside the egg). The number of eggs is the sum of hatched, embryonated, and unembryonated eggs (egg in the morula stage, without miracidium).

The data were analyzed using the statistical computer package for social sciences SPSS. The independent sample t-tests and two-way ANOVA tests were used to assess the differences between groups. A *P*-value of less than 0.05 was considered significant ($p<0.05$). In this study, a small number of outliers with results far from the dominant mean population were excluded from the study.

PCR-RFLP. The genomic DNA extraction from specimens was performed using a commercial kit (Genomic DNA Extraction SynBitech Miniprep Kit) and stored at-20ºC until used. A ~460 bp fragment spanning the 18S rDNA and first internal transcribed spacer (ITS1) regions of *Fasciola* species was amplified using the primers (FascF: 5′-ACC GGT GCT GAG AAG ACG-3′ and FascR: 5′-CGA CGT ACG TGC AGT CCA-3′) previously used by others [18]. PCR mixture was prepared in 25 µL volumes containing 10.5 µl DW, 12.5 µl of 2× premix (Ampliqon, Skovlunde, Denmark), 0.2 µM of each primer, and 1 µl extracted DNA. The amplification was performed in a thermocycler (A&E Lab (UK) Co., Ltd) programmed for an initial denaturation at 95 ˚C for 5 min followed by 30 cycles at 95˚C for 45 s, 60 ˚C for 45 s, and 72 ˚C for 1 min. PCR products were visualized on 1.5 % agarose gel using a UV transilluminator. The amplicons were subjected to restriction fragment length polymorphism (RFLP) using the enzyme *TasI* (Thermo scientific). The digestion was carried out in 15 µl reaction mixtures containing 5 µl of Fasciola ITS1-PCR product, 8 µl DW, 0.5 µl TasI, and 1.5 µl of 10× supplied buffer. The mixtures were then incubated at 65ºC for 2.5 h, followed by visualizing the digested products on 2% agarose gels in a UV transilluminator [18].

RESULTS

Egg hatch assay. The eggs of both *Fasciola* species were yellowish and oval with an operculum at one of the endpoints. One embryonic cell mass was surrounded by many yolk cells in the middle line of the eggs. The eggs from the same fluke varied in size. All eggs, hatched, embryonated, and unembryonated, were counted (Fig. 1). The percentages of hatched eggs of *F. gigantica* and *F. hepatica* species originated from sheep were 31.69% and 32.59%, respectively, and from the cattle were 69.19% and 62.36%, respectively (Table 1). Also, there were considerable differences in the percentage of hatching eggs between sheep and cattle ($P\leq0.05$). In contrast, this relationship was not significant with the parasitic species ($P\leq0.05$). On day 15 of incubation,

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the percentage of developed eggs in *F. gigantica* and *F. hepatica* from sheep were 69.32% and 72.71%, respectively, and from cattle were 73.56% and 74.69%, respectively (Table 1). In this study, no significant difference was observed in developed eggs percentages regarding animal or parasite species ($P \leq 0.05$).

Table 1. The development of *F. hepatica* and *F. gigantica* eggs in sheep and cattle-source under 26±1 ºC and 15 days of incubation

		Parasite species							
Host species		***Fasciola hepatica***				***Fasciola gigantica***			
	Eggs	**Samples**			**Average (%)**	**Samples**			**Average (%)**
Sheep	**Undeveloped**	286	265	163	238 (27)	210.6 (30)	350	203	210.6 (30)
	Embryonated	356	377	317	350 (40)	258.3 (37)	307	335	258.3 (37)
	Hatched	287	299	267	284.3 (32)	217.6 (31)	215	312	217.6 (31)
	Hatched (%)	30.9	31.7	35.7	32.8	32.87	24.6	36.7	32.87
	Developed (%)	69.2	71.8	78.4	73.16	70.86	59.8	76.1	70.86
Cattle	**Undeveloped**	105	46	165	105.3 (25)	123 (25)	151	134	123 (25)
	Embryonated	29	35	90	51.3 (12)	20.3 (41)	25	21	20.3 (41)
	Hatched	178	143	458	259.6 (62)	322 (66)	340	419	322 (66)
	Hatched (%)	57.0	63.8	64.2	61.7	64.99	65.8	72.9	64.99
	Developed (%)	66.3	79.4	76.8	74.21	69.18	70.7	76.6	69.18

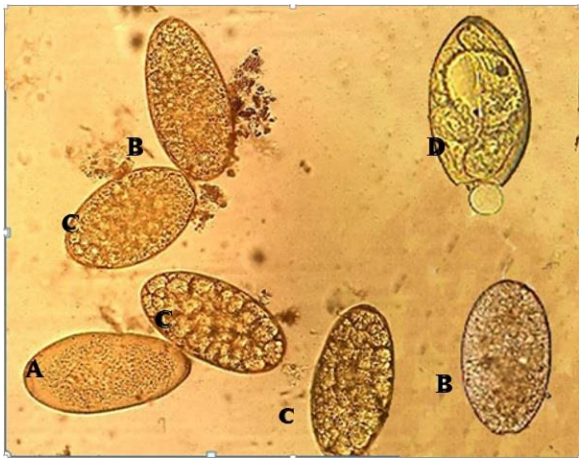


Fig 1. Light micrographs illustrating the different stages of egg development. (A) Dead eggs; (B) unembryonated egg; (C) eggs at cell division; (D) eggs at eyespot stage.

PCR-RFLP. A ≈ 460-bp amplicon was amplified in all *F. hepatica* and *F. gigantica* samples (Fig. 2A); no amplification was obtained with negative controls. Digesting the PCR products with *Tas1* revealed two patterns: one with two bands (150 bp and 310 bp) indicating *F. hepatica* and another with three bands (95bp, 150bp 220bp) indicating *F. gigantica* (Figure 2B). The PCR-RFLP analysis corroborated morphological results.

DISCUSSION

In the current study, the hatching rates of *F. gigantica* and *F. hepatica* originated from cattle were higher than those from sheep, while there was no change in the percentage of developed eggs in the two hosts. Miracidia maturation occurs within 12–16 days in eggs of both *F. hepatica* and *F. gigantica* regardless of the host species [16]. Several reports showed that the chemical

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composition of various plant extracts [19] could penetrate the eggshell and inhibit the egg hatching depending on the solubility in lipids [20]. Also, albendazole and triclabendazole ovicidal activity against *F. hepatica* eggs and the inhibitory effects of these compounds on the hatching process has been observed [21].

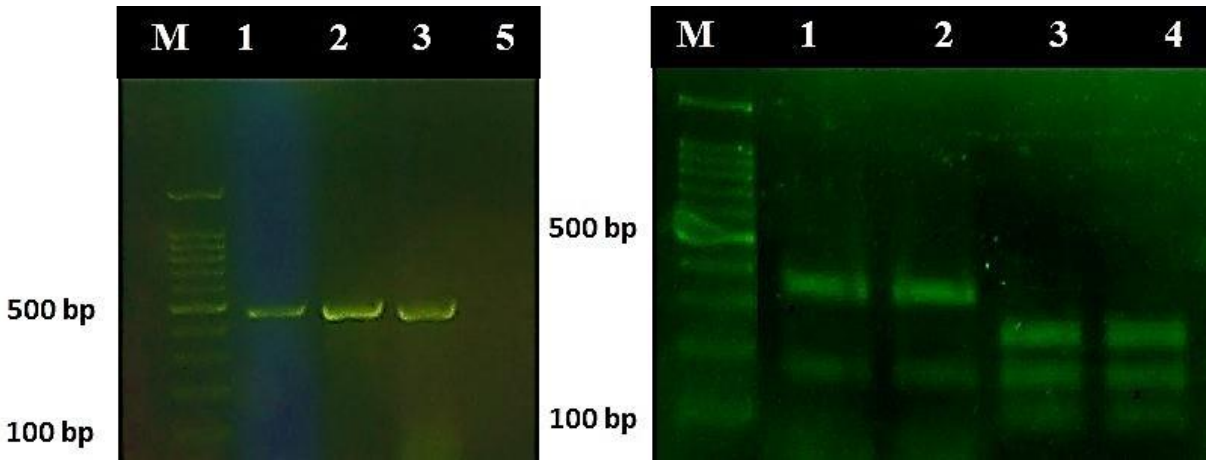


Fig 2. PCR-RFLP analysis of the ITS-1 region for diagnosing *Fasciola* species. A) PCR amplification of ITS1 fragment, B) digestion of amplicons with *TAS-1* enzyme; lanes 1-2, *F. hepatica*; lanes 3-4, *F. gigantica*; lane 5, negative control; lane M, a 100 bp DNA marker (CinnaGen Inc., Iran).

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The percentages of ovine hatched and developed *F. hepatica* eggs in the current study were 32.8% and 73.16%, respectively, similar to the results reported by Robles-Pérez et al. (2014) [22]. In their study, the percentages of hatched and developed eggs of the susceptible isolates of ovine *F. hepatica* to albendazole were 33% and 72%, respectively, 14 days post-incubation at 25ºC in darkness [22]. In another study, the percentage of hatched eggs in the control group of triclabendazole-resistant isolates of ovine *F. hepatica* was 33%, consistent with our results in the same species [23].

In our study, the development and hatching percentage of bovine *F. gigantica* eggs were higher than those of the same species in sheep (69.18% vs. 64.99%) at 26 ◦C, which agrees with the results obtained from untreated *F. gigantica* eggs taken from cattle in a similar study under incubation at 28°C for 14 days (82% ± 2 and 73% ± 2) [24]. The slight difference between their results and our findings may be related to the high incubation temperature in their study. Furthermore, the incubation period of eggs affects their hatching rate; Al-jibouri et al. (2010) reported that the percentage of hatched *F.*

composition of various plant extracts [19] could penetrate the eggshell and inhibit the egg hatching depending on the solubility in lipids [20]. Also, albendazole and triclabendazole ovicidal activity against *F. hepatica* eggs and the inhibitory effects of these compounds on the hatching process has been observed [21].

gigantica eggs originated from cattle on day 17 post-incubation at 25ºC was 73.3% [25].

In addition to the host role, environmental factors such as light, temperature, pH, sodium chloride concentration [26, 27], and incubation period affect the development and hatching of *Fasciola* eggs. Moazeni et al. (2010) reported that the percentage of developed *F. hepatica* eggs on day 16 of incubation at 28ºC was 52% without interfering with the host role, which was different from our results [13]. Our findings indicated that the percentage of developed *F. hepatica* eggs under the same conditions was higher than that of *F. gigantica* eggs in both cattle and sheep hosts. In this study, we did not perform micrometry of the eggs; however, the average size for *F. gigantica* eggs was larger (150–196/90–100 µm) than those of *F. hepatica* (30–150/63–90 µm) [8], and it was recently proven the host species affects this parameter [28]. A study in northwestern Iran showed a significant difference between the length, width, and proportion of length to width of the *F. hepatica* eggs originated from sheep and cattle. The length to width ratio in *F. hepatica* eggs from sheep (1.65) was more prominent than from cattle (0.23) [12]. Unlike previous reports, the average *F. hepatica* egg size in sheep was

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bigger than that of *F. gigantica* (233.8±19.9/141.8±16.0 µm vs. 230.7±16.2 /138.1 ±12.76 µm). Also, *F. hepatica* eggs in cattle were bigger than those of *F. gigantica* (242.3±15.1/135.3±14.4 µm and 229.9±15.3/130.7±13.5 µm [12]. The genetic nature of native parasites may influence morphometric characteristics of *Fasciola* eggs reported in these studies. The hatching period for *F. hepatica* was 13-15 days and shorter than 12-16 days of *F. gigantica* [16]. Therefore, on day 15 of incubation, most *F. hepatica* eggs developed earlier than *F. gigantica*, as seen in the present study. Faster development of *F. hepatica* eggs may be due to the difference in operculum shapes of eggs. The operculum of *F. gigantica* eggs has a distinct inner concave shape, while *F. hepatica* eggs have a thin, short, and straight/flat operculum [16]. The hatching of *F. hepatica* eggs starts with pushing miracidia to the narrow and partially opened operculum alongside proteolytic hatching enzyme, while the shell-opercular attachment of *F. gigantica* eggs begins to loosen and cracks on one side [16, 28]. Hussein et al. (2010) showed that the definitive host species affects the size of *F. hepatica* eggs and adult worms [16]. Mendes et al. (2008) reported that the *F. hepatica* eggs derived from cattle were significantly bigger than those from marmoset [29]. However, no difference in the size of *Fasciola* spp. eggs was observed in a mouse model [30]. Thus, the structure of shell-operculum of *Fasciola* spp. eggs and the different sizes of *Fasciola* spp. (parasites specificity) can affect the percentage of developed eggs during the hatching process. The miracidia hatching of *Echinostoma caproni* eggs occurred on day 11 at 27ºC in hamster-derived eggs, while this period was 13 days for eggs originated from mice [14]. The maturation of miracidia within eggs occurred in 9 and 7-8 days after removing adult trematodes from the small intestine of hamsters and mice, respectively [14]. Therefore, the host species can influence the formation and development of miracidia as well as the hatching rate of trematode eggs.

Moreover, fasciolosis pathogenesis and clinical symptoms vary in severity, from a destructive disease in sheep, alpacas, and llamas to an asymptomatic infection in cattle [31]. The difference in the pathogenesis of fasciolosis in cattle and sheep significantly shows the importance of the host role in the eggs hatching process. The present study results showed that the *Fasciola* egg hatching rate in cattle was higher than in sheep, without significant changes in the rate of developed eggs in different hosts.

Lambert et al. (2015) investigated the hatching rate and volume of *Trichostrongylus retortaeformis* and *Graphidium strigosum* eggs shed in infected rabbit feces concerning the host immune responses. A two-week follow-up showed that the host antibodies play a minor role in the egg hatching rate of these gastrointestinal helminths [1]. In the present study, we studied intrauterine eggs; hence, hatching is more affected by host responses to female worms than by direct response to eggs, though some host characteristics such as various bile compositions in different mammals [32] on female worms should not be ignored. Clarifying various biological behaviors in parasites can help to identify the pathogenic pattern of the disease in different hosts and improve success in parasitic control and prevention programs. Our study enhanced our understanding of the variations in transmission and epidemiological characteristics of these two parasites in ruminants.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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